

# Increased Expression of Two Alternative Spliced Variants of CD1d Molecule in Human Gastric Cancer

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## ABSTRACT

**Background:** CD1d presents glycolipid antigens to invariant natural killer T (iNKT) cells. The role of CD1d in the development of peptic ulcer and gastric cancer has not been revealed, yet. **Objective:** To clarify the expression of alternatively spliced variants of CD1d in peptic ulcer and gastric cancer. **Methods:** Patients with dyspepsia were selected and divided into three groups of non-ulcer dyspepsia (NUD), peptic ulcer disease (PUD), and gastric cancer (GC), according to their endoscopic and histopathological examinations. *H. pylori* infection was diagnosed by rapid urease test and histopathology. The expression levels of V2, V4, and V5 spliced variants of CD1d molecule were determined by quantitative Reverse Transcriptase PCR. **Results:** Relative gene expression levels of V4 were higher in GC patients (n=37) than those in NUD (n=49) and PUD (n=51) groups (p<0.05 and p<0.01, respectively). Moreover, GC patients showed higher expression levels of V5 compared to NUD and PUD groups (p<0.001 and p<0.001, respectively). Positive correlation coefficients were attained between V4 and V5 expression in patients with PUD (r=0.734, p<0.0001) and GC (r=0.423, p<0.01), but not in patients with NUD. Among NUD patients, the expression levels of V4, but not V5, were higher in *H. pylori*-positive patients than in *H. pylori*-negative ones (p<0.01). **Conclusion:** Collectively, both membrane-bound (V4) and soluble (V5) isoforms of CD1d were over-expressed in gastric tumor tissues, suggesting that they are involved in anti-tumor immune responses.

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## INTRODUCTION

Peptic ulcer disease (PUD) and gastric cancer (GC) are common diseases worldwide (1,2). *Helicobacter pylori* has been recognized as a main risk factor for the development of PUD and GC (3,4). Most of the infected individuals (80-90%) carry and spread the bacterium while they are asymptomatic, while others develop one of the two clinical outcomes, PUD and GC. The reasons for developing these two extreme outcomes have not been clearly understood (4). It has been reported that colonization of *H. pylori* in human gastric mucosa activates innate immune responses by gastric epithelial cells which can lead to adaptive immune responses (5). Moreover, interaction between *H. pylori* surface antigens and receptors of innate immune cells inside the gastric mucosa might determine the pathological outcome of *H. pylori* infection, including development of PUD or GC (5). On the other hand, *H. pylori* can evade the immune system via suppression of innate immune responses; this might lead to active survival of bacteria in local mucosal environment, which, in turn, affects the development of mentioned pathological conditions (6). Therefore, study of cells and molecules involved in innate immunity can lead to better understanding of innate immune circumstances resulting in peptic ulcer and gastric cancer.

Invariant Natural Killer T (iNKT) cells are innate T lymphocytes that express an invariant TCR  $\alpha$  chain and recognize glycolipid antigens mostly presented by CD1d molecules (7). The frequency of iNKT cells in human gastric mucosa has been shown to be higher in *H. pylori* infected subjects compared to non-infected individuals (8). Moreover, one study showed that cholesteryl  $\alpha$ -glucosides, which constitute 25% of total *H. pylori* lipids, can be bound with CD1d, and induce an immune response by iNKT cells, thus causing inflammation in gastric mucosa (9).

CD1d molecule is a membranous glycoprotein which has many structural similarities with histocompatibility class 1 (MHC-I) genes, both encode transmembrane proteins type 1 ( $\alpha$  heavy chain) with three extracellular domains,  $\alpha 1$ ,  $\alpha 2$ , and  $\alpha 3$ , associated non-covalently to  $\beta 2$  microglobulin ( $\beta 2M$ ) (10). CD1d is expressed on a variety of immune cells including dendritic cells, B cells, and T cells (10), as well as on different epithelial cells (11-13), where it presents glycolipid antigens to innate immune cells, mainly iNKT cells (14). Benam *et al.* investigated the expression of the six alternatively spliced variants of CD1d in human bronchial epithelial cells and showed that these variants are specific to epithelial cells (15). Among these alternative spliced variants of CD1d, only V2, V4, and V5 seem to be able to bind antigens because they preserve their antigen binding domains ( $\alpha 1$  and  $\alpha 2$ ) (Figure 1). According to that study, V2 is deficient in the trans-membrane (TM) domain, while V4 and V5 lack  $\alpha 3$  and  $\alpha 3$ -TM domains, respectively (15). Therefore, V2 and V5 represent the soluble forms, while V4 represents the membranous form (15). In the present study, V2, V4, and V5 were selected to be investigated, since, all three variants contain intact  $\alpha 1$  and  $\alpha 2$  domains forming antigen binding region, thereby likely capable of presenting glycolipid antigens.

Since CD1d is an innate immune receptor on different epithelial cells which can recognize *H. pylori* lipid antigens, and given the role of *H. pylori* recognition by gastric epithelial cells in the development of mucosal inflammation and subsequent occurrence of gastritis, gastric ulcer, and gastric cancer, we hypothesized that there might be an association between the expression levels of CD1d spliced variants and development of dyspeptic disorders in *H. pylori* infected individuals. Therefore, this study aimed to

determine the expression levels of V2, V4, and V5 isoforms of CD1d molecule in gastric biopsy specimens of *H. Pylori*-positive and-negative patients with dyspeptic disorders, including non-ulcer dyspepsia (NUD), PUD, and GC.

## MATERIALS AND METHODS

**Patients.** Patients with dyspepsia, who underwent esophago-gastro-duodenoscopy at Imam Hospital or the Outpatient Clinic of Mazandaran University of Medical Sciences (Sari, Iran), were enrolled in the study. All samples were taken between January 2012 and December 2013. The study was approved by the Ethics Committee of Mazandaran University of Medical Sciences. Clinical history, demographic data, and written informed consent were taken from all study subjects. None of the subjects had a history of chronic inflammatory or autoimmune disorders, received *non-steroidal anti-inflammatory drugs (NSAIDs)* during past two weeks, or had a history of *H. pylori* eradication therapy. None of patients with GC had received surgery, radiotherapy, chemotherapy, or any other form of medical interventions before sample collection. Three tissue samples from the gastric antrum were taken from each patient during endoscopy. One of the tissue samples was applied for the rapid urease test and the second one was fixed and processed for routine histopathological examination. Based on the endoscopic and histopathological assessments, samples were divided into three groups of non-ulcer dyspepsia (NUD), peptic ulcer disease (PUD), and gastric cancer (GC). The histological grade of the gastric tumors was determined on the basis of differentiation. The presence of *H. pylori* infection was determined by the rapid urease test and histopathological examination (including Giemsa staining). Patients were considered as *H. pylori*-positive if the results by at least one test was positive and *H. pylori*-negative if the results by both tests were negative.

**Table 1. Characteristics of the study subjects.**

		NUD (n=52)	PUD (n=53)	GC (n=39)
Age (mean ± SD)		47.1 ± 14.6	54.4 ± 18.5	71.4 ± 10.6
Sex	Male	13 (25.0%)	24 (45.3%)	32 (82.1%)
	Female	39 (75.0%)	29 (54.7%)	7 (17.9%)
<i>H. pylori</i>	Positive	30 (57.7%)	36 (67.9%)	20 (51.3%)
	Negative	19 (38.8%)	16 (30.2%)	17 (43.6%)
	ND	3 (5.8%)	1 (1.9%)	2 (5.1%)
Tumor Grade	G1			3 (7.7%)
	G2			11 (28.2%)
	G3			20 (51.3%)
	G4			0 (0%)
	ND			5 (12.8%)

NUD: Non-ulcer dyspepsia, PUD: Peptic ulcer disease, GC: Gastric cancer, ND: Not defined

Patients in each of the three groups were then divided into two subgroups of *H. pylori*-positive and -negative. Table 1 shows the demographic data of patients as well as the histopathologic grading of gastric tumors.

The third tissue sample from each patient was preserved in the RNase inactivating solution (RNAlater, Qiagen, Germany) for RNA extraction.

**RNA Isolation and cDNA Synthesis.** Each tissue specimen was homogenized using mortar and pestle at room temperature. Total RNA was extracted from 50 mg of dissected tissues using a commercial RNA extraction kit (RNeasyMinikit; Qiagen, Germany), according to the manufacturer's instructions. The quantity and quality of extracted RNAs were assessed by a nanodrop spectrophotometer (Thermo Fisher Scientific Inc., US) and agarose gel electrophoresis, respectively. RNA (1µg) was reverse-transcribed into complementary DNA (cDNA) using the RevertAid<sup>TM</sup> First-Strand cDNA Synthesis Kit (Fermentas, Germany) primed with random hexamer primer as per the manufacturer's instructions.

**Primer Designing.** The sequences of CD1d and Hypoxanthine-guanine phosphoribosyl-transferase (HGPRT), as a normalizer, was obtained from the GenBank (Table 2). Primers for amplification of HGPRT as well as V2, V4, and V5 spliced variants of CD1d were designed using the Beacon designer software and synthesized by TIBmol (Germany) (Table 2).

**Table 2. Primers and probes used for real-time PCR quantification.**

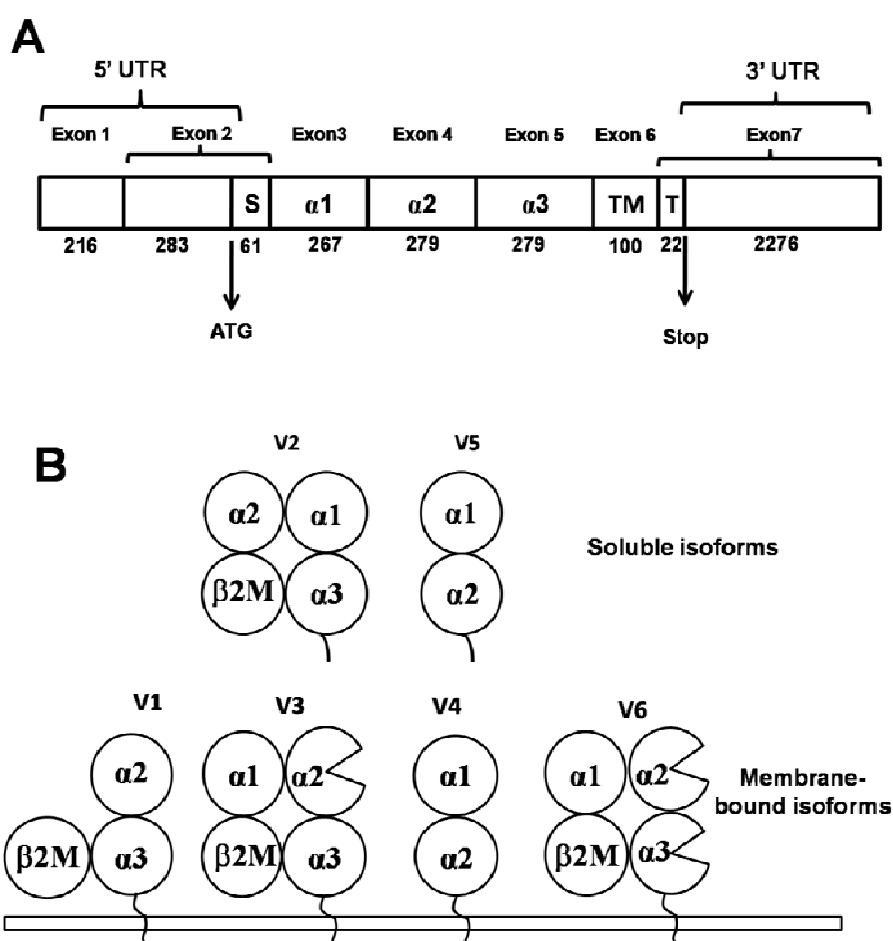
Gene	Genbank Accession Number	Primers (5'-3')*	Product Size (bp)
CD1d	NM_001766	<b>For. V2</b> , GAA CTG AAG AAG CAA GTG AAG <b>Rev. V2</b> , CCT GAT AGG AAC CCA GTA GAG	306
		<b>For. V4</b> , GCT ACG CTT ATC CTA TCC CTT G <b>Rev. V4</b> , AGC TCC CAC CTT GCT TCT TC	302
		<b>For. V5</b> , GCT ACG CTT ATC CTA TCC CTT G <b>Rev. V5</b> , GCC CTG ATA GGA ACT TGC TTC	306
HGPRT	NM_000194.2	<b>For.</b> , CTA ATT ATG GAC AGG ACT GAA CG <b>Rev.</b> , TTG ACT GGT CAT TAC AAT AGC TC	211

\* For., forward primer; Rev., reverse primer.

Based on Benam *et al.* (15), V2 lacks the trans-membrane (TM) domain, which is encoded by exon 6. Thus, the primers for V2 were designed to amplify the entire exon 5 along with the junction of exon 5 to exon 7. On the other hand, V4 lacks  $\alpha 3$  domain encoded by exon 5. Therefore, the primers for V4 were designed to amplify the whole exon 4 plus the junction of exon 4 to exon 6. The primers for V5 amplification were designed to amplify the whole exon 4 plus the junction of exon 4 to exon 7, as V5 is deficient in  $\alpha 3$  and TM domains encoded by exons 5 and 6, respectively (Figure 1).

**Quantitative Reverse Transcriptase Polymerase Chain Reaction.** Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was performed using 96 well plates (Bio-Rad Laboratories Inc., USA) in a volume of 20 µL containing Maxima

SYBR Green/ROX qPCR Master Mix (2X) (Thermo Scientific), 10 pmols of each of forward and reverse primers, and 2  $\mu$ L of cDNA. The samples were denatured at 95°C for 10 min, and amplified using 40 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30s on an iQ5 real-time thermal cycler (Bio-Rad Laboratories Inc.). Ct (cycle of threshold) values corresponding to the number of PCR cycles at which the fluorescence emission monitored in real time exceeded a threshold limit (10 $\times$  the standard deviation of the baseline intensity) were measured. A mean cycle of threshold (Ct) value for each duplicate measurement was calculated. Relative gene expression was then calculated using “ $\Delta$ Ct method using a reference gene” in the following manner for each sample: Ratio (reference/target) =  $2^{Ct(\text{reference}) - Ct(\text{target})}$



**Figure 1.** Graphical representation of CD1d mRNA and alternative spliced CD1d isoforms. The CD1d heavy chain contains  $\alpha 1$ ,  $\alpha 2$ ,  $\alpha 3$ , transmembrane (TM), and cytoplasmic tail domains, which are encoded by chromosome 1 and a light chain  $\beta 2M$  molecule which is encoded by chromosome 15. Exons 3-5 encode  $\alpha 1$ - $\alpha 3$  domains, respectively. The antigen-binding site is composed by exons 3 and 4. V1 to V6 are six alternatively spliced isoforms of CD1d transcripts. V2 and V5 lack the TM domain and are thus represented as soluble isoforms. In contrast, V1, V3, V4, and V6 contain TM domain and are represented as membrane-bound isoforms.  $\alpha 2$  in V3 and  $\alpha 2$  and  $\alpha 3$  in V6 are incomplete domains and thus illustrated with incomplete circles (This figure is illustrated based on information adopted from Benam *et al.* (15)).

**Statistical Analysis.** Statistical analysis was performed using the SPSS statistical package (SPSS, Chicago, IL, USA). The results were evaluated by independent-samples *t*-test, Mann-Whitney U test and Pearson and Spearman correlation tests where appropriate. The strength of association between the expression levels of CD1d variants was analyzed using the Spearman rank order correlation test, and the correlation coefficient was calculated. Findings were considered significant when p-values were <0.05. The results presented in the text and tables represent geometric mean in case of  $2^{\Delta Ct}$  and mean  $\pm$  standard deviation (SD) in case of other variables.

## RESULTS

Forty nine patients with NUD, 51 with PUD, and 37 with GC were enrolled in this study (Table 1).

**Increased Expression of V4 and V5 in GC Patients.** In order to measure the expression levels of V2, V4, and V5, cDNA from each tissue sample was individually amplified using the primer pairs for V2, V4, and V5 as well as for HGPRT as a normaliser. Relative expression levels of V2, V4, and V5 were then calculated by “ $\Delta Ct$  method, using a reference gene” as mentioned above. As Figure 2 shows, relative expression levels of V4 were higher in GC patients than those of NUD and PUD groups ( $p < 0.05$  and  $p < 0.01$ , respectively) (Figure 2 and Table 3).

**Table 3. Relative mRNA expression levels of CD1d spliced variants, V4 and V5, in each group of patients.**

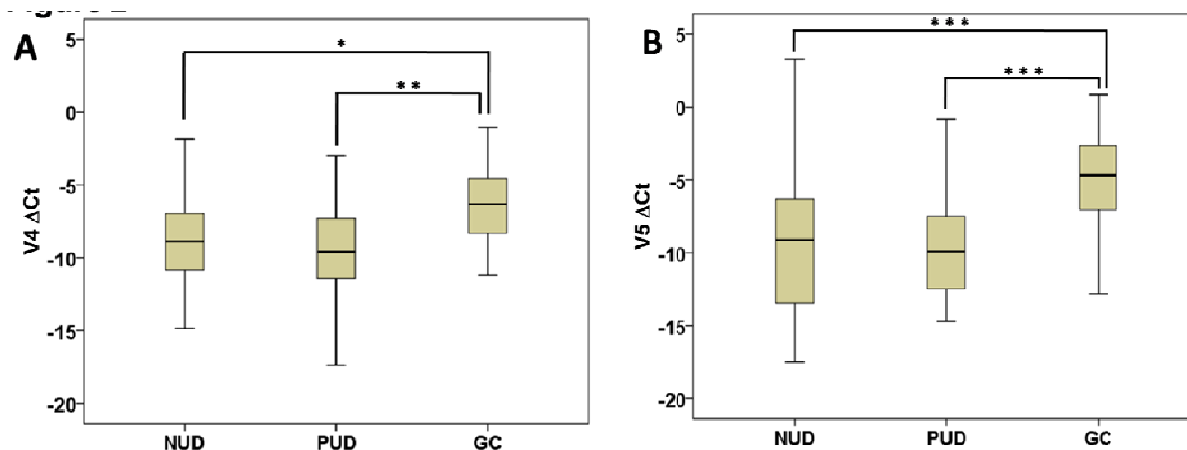
CD1d Isoform	Group	Number of Samples	Relative mRNA Expression		P Value
			$\Delta Ct$ (Mean)	$2^{\Delta Ct}$ (Geometric mean)	
V4	NUD	49	-8.54	0.0027	<0.05*
	PUD	51	-8.79	0.0022	<0.01**
	GC	37	-6.32	0.0125	
V5	NUD	48	-9.42	0.0015	<0.001***
	PUD	51	-9.32	0.0016	<0.001***
	GC	37	-4.92	0.0330	

\* Relative expression levels of V4 in NUD patients were significantly lower than those in GC group ( $p < 0.05$ ).

\*\* Relative gene expression levels of V4 in PUD patients were significantly lower than those in GC group ( $p < 0.01$ ).

\*\*\* Relative gene expression levels of V5 in both NUD and PUD patients were significantly lower than those in GC group ( $p < 0.001$ ).

Moreover, GC patients showed higher expression levels of V5 compared to NUD and PUD groups ( $p < 0.001$  and  $p < 0.001$ , respectively) (Figure 2 and Table 3). There was no significant difference between NUD and PUD groups regarding V4 or V5 expression levels. Amplification plots of quantitative real-time PCR with designed primers for V2 produced late Ct on and were inconsistent. Therefore, we failed to determine the relative expression levels of V2.



**Figure 2.** Relative gene expression levels ( $\Delta$ CT) of CD1d spliced variants, V4 (A) and V5 (B), in patients with NUD, PUD, and GC.

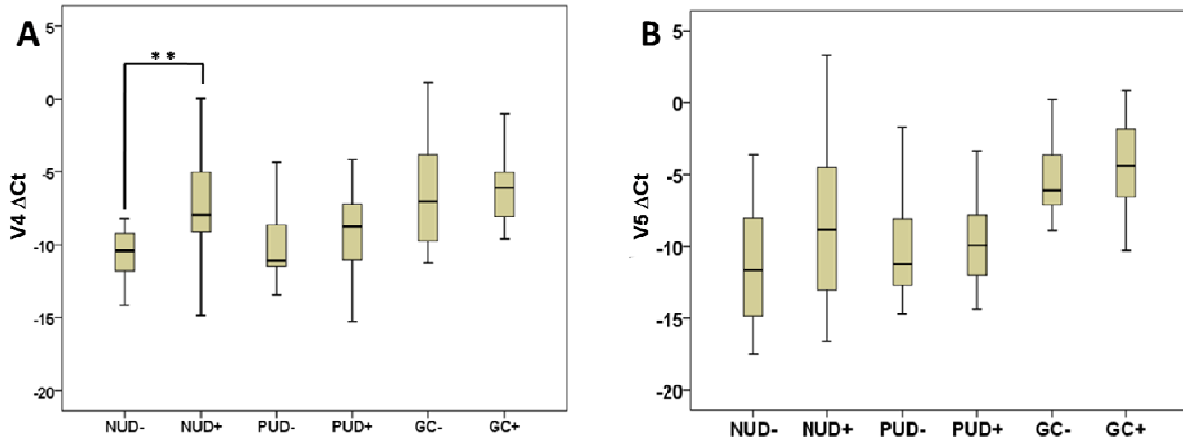
**Increased Expression of V4, but not V5, in *H. pylori*-positive NUD Patients.** Relative expression levels of V4 and V5 were further analyzed between *H. pylori*-positive and -negative patients in each group of NUD, PUD, and GC. The results showed that among NUD patients, relative gene expression levels of V4 in *H. pylori*-positive patients were significantly higher than those in *H. pylori*-negative ones ( $p < 0.01$ ) (Figure 3A and Table 4).

**Table 4. Relative mRNA expression levels of CD1d spliced variants, V4 and V5, in each group of patients regarding *H. pylori* infection.**

CD1d Isoform	Group	<i>H. pylori</i>	Number of Samples	Relative mRNA Expression		P Value
				$\Delta$ CT (Mean)	$2^{\Delta$ CT (Geometric mean)	
V4	NUD	Positive	30	-7.62	0.0050	0.004**
		Negative	19	-10.07	0.0009	
	PUD	Positive	36	-8.21	0.0034	0.119
		Negative	15	-9.99	0.0010	
	GC	Positive	20	-6.24	0.0132	0.715
		Negative	17	-6.35	0.0122	
V5	NUD	Positive	29	-8.58	0.0026	0.084
		Negative	19	-11.21	0.0004	
	PUD	Positive	36	-9.13	0.0018	0.444
		Negative	15	-10.02	0.0010	
	GC	Positive	20	-4.43	0.0464	0.345
		Negative	17	-5.41	0.0234	

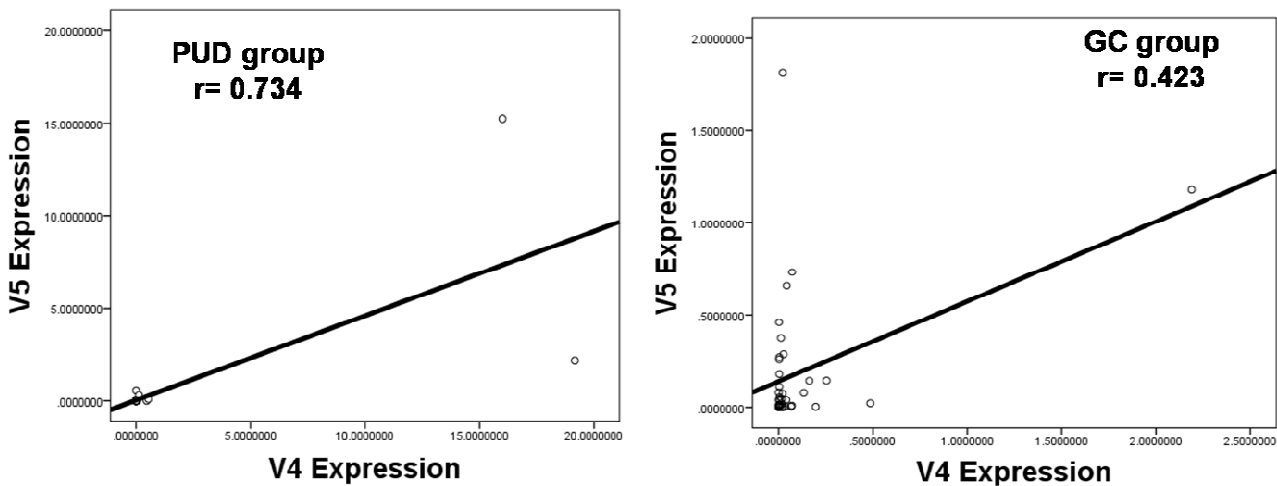
\*\* The expression levels of V4 were significantly higher in *H. pylori*-positive NUD patients than in *H. pylori*-negative ones ( $P < 0.01$ ).

However, there was no significant difference between *H. pylori*-positive and -negative patients in PUD and GC groups regarding V4 expression (Figure 3A and Table 4). Moreover, the expression levels of V5 were not significantly different between *H. pylori*-positive and -negative patients in none of NUD, PUD, or GC groups (Figure 3B and Table 4).



**Figure 3.** Relative gene expression levels ( $\Delta$ Ct) of CD1d spliced variants, V4 (A) and V5 (B) in *H. pylori*<sup>+</sup> and *H. pylori*<sup>-</sup> patients with NUD, PUD, and GC.

**Positive Correlations between V4 and V5 Expression.** The strength of association between the expression levels of V4 and V5 was further analyzed using the Spearman rank order correlation test, and the correlation coefficient was calculated.



**Figure 4.** Linear Correlations between the expression levels ( $\Delta$ Ct) of V4 and V5 in patients with PUD ( $r=0.734$ ,  $p<0.0001$ ) and GC ( $r=0.423$ ,  $p<0.01$ ).



Positive correlation coefficients were attained between V4 and V5 expression in patients with PUD ( $r=0.734$ ,  $p<0.0001$ ) and GC ( $r=0.423$ ,  $p<0.01$ ) but not in patients with NUD (Figure 4).

## DISCUSSION

The present study showed that the expression levels of two alternatively spliced variants of CD1d, V4 and V5, were significantly higher in patients with gastric cancer than those with non-malignant inflamed gastric mucosa, NUD and PUD. Moreover, the expression levels of V4 and V5 were significantly correlated with each other in GC patients. Previous studies on the expression of CD1d on the surface of tumor cells and its role in the presentation of tumor antigens have reported opposing results. Some studies have reported an up-regulation of CD1d in several types of tumors, such as glioma (16), medulloblastoma (17), prostate Cancer (18), and some types of leukemia (19,20). Moreover, tumor cells expressing functional CD1d have been targeted for direct NKT-cell cytotoxicity using either synthetic NKT ligands or ex-vivo expanded NKT cells (21). For instance,  $\alpha$ -GalactosylCeramide ( $\alpha$ -GalCer), as a synthetic ligand, can stimulate NKT cells in a CD1d dependent manner, which results in production of Interferon (IFN)- $\gamma$  and Interleukin (IL)-4, demonstrating  $\alpha$ -GalCer as an important antitumor stimulator in different tumor models (22).

In contrast, some other studies have reported that the majority of solid tumors were CD1d negative (23). One study showed the down-regulation of CD1d on breast cancer cells, which led to inhibition of iNKT cell-mediated antitumor immunity, and promoting breast cancer metastasis (24). Another study revealed the lower expression levels of CD1d by omental hematopoietic cells and the lower number of iNKT cells in colon cancer patients compared to healthy controls (25). Moreover, down-regulation of CD1d molecule in patients with malignant lymphoma has led to reduced expansion of iNKT cells (26). Therefore, it seems that up- or down-regulation of CD1d molecule affects anti-tumor iNKT cell response in different ways. In the present study, in contrast to the previous studies, three alternative spliced variants of CD1d, including V2, V4, and V5, were selected to be investigated, since all three variants contain intact  $\alpha 1$  and  $\alpha 2$  domains which form antigen-binding region making these variants capable of presenting glycolipid antigens. If we assume that CD1d-associated presentation of tumor antigens, such as GD3 and glycosphingolipids, to iNKT cells can lead to an anti-tumor immune response, how can the gastric tumor develop while both V4 and V5 are over-expressed in the gastric mucosa? One explanation could be that although up-regulation of V4, as a membranous form of CD1d presenting tumor associated glycolipid antigens, leads to iNKT cell activation, simultaneous binding of V5, as a soluble form of CD1d, to the same antigens, might act as a competitive inhibitor for the V4. Therefore, up-regulation of V5 might be a mechanism by which tumor cells could evade the innate immune system. On the other hand, Kojo *et al.* in 2000 reported that variants of CD1d without  $\alpha 3$  domain might be unstable presented on the cell surface (27). Therefore, given that V4 lacks  $\alpha 3$  domain, it might be unstably presented on the surface of gastric epithelial cells, which, in turn, leads to inadequate activation of NKT cells. Another explanation could be that over-expression of CD1d variants does not necessarily lead to increase iNKT cell responses against tumor, as many studies have shown that the number and function of these cells were reduced in the several types of cancers (18,19,28).

We further compared the expression levels of V4 and V5 between *H. pylori*-positive and-negative samples in each group of NUD, PUD, and GC. The results showed that the expression levels of V4, but not V5, were significantly higher in *H. pylori*-positive NUD patients than those in *H. pylori*-negative ones. In contrast to NUD patients, comparison of the expression levels of V4 and V5 in *H. pylori*-positive and -negative PUD and GC patients showed no significant difference. Therefore, the over-expression of V4, as a membranous isoform of CD1d, in *H. pylori*-positive NUD patients might be a mechanism to increase presentation of bacterial lipid antigens to iNKT cells and stimulates innate immune responses against infection. In this regard, it has been shown that cholesteryl  $\alpha$ -glucoside, the major component of *H. pylori* cell wall lipids, could interact with CD1d and contribute to induction of iNKT cell responses (9) suggesting that CD1d is able to present *H. pylori* antigens to iNKT cells. Under malignant circumstance of gastric cancer, however, it seems that the over-expression of CD1d variants is independent of *H. pylori* infection. Probably, tumor antigens, like tumor-associated altered glycolipid ligands, dominate *H. pylori* antigens in iNKT cell activation. Concurrently, GD3, a ganglioside that is highly expressed by human tumors of different origins, has been shown to be recognized by mouse NKT cells in a CD1d-restricted manner (29). In addition, CD1d has been reported to be involved in the presentation of inflammation-associated lyso-phospholipids from the plasma of myeloma patients to CD1d-restricted T cells (30).

We failed to determine the relative expression levels of V2, as V2 produced late Ct on amplification plot and was inconsistent. We believe that this shows very low levels of V2 expression, as it was also reported by Benam *et al.* (15).

In summary, this study, for the first time, determined the expression levels of the three alternative spliced variants of CD1d, which probably preserved their antigen-binding groove, in human gastric mucosa. An over-expression of both membranous isoforms, V4, and soluble isoform, V5, of CD1d was observed in gastric tumor tissues. Moreover, the expression levels of V4 and V5 were significantly correlated with each other in GC patients. We thus hypothesize that although the membranous form of CD1d can present glycolipid antigens and stimulates an anti-tumor immune response, the soluble form binds to iNKT cell receptors and finally inhibits the iNKT cell response. We also found out a V4 over-expression in *H. pylori*-positive NUD patients compared to *H. pylori*-negative ones suggesting the role of V4 in presentation of *H. pylori* antigens in inflamed gastric mucosa. In gastric tumor samples, however, CD1d expression seems to be independent of *H. pylori* infection, since it is implicated predominantly in the presentation of altered glycolipid ligands associated with tumor. In future, we suggest investigating the role of different variants of CD1d in proliferation of iNKT cells *in vitro*, and also possible correlations between the expression levels of CD1d spliced variants and the frequency and function of iNKT cells in gastric epithelial layer.

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